An Investigation of a Sonochemical Approach in Sterilization Problems

Fifth Semiannual Progress Report

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1. Purpose of Investigation.

Studies on the effects of ultrasonic irradiation and propylene oxide, used in combination, against spores of Bacillus subtilis var. niger have continued. The results of experiments performed since the fourth semiannual progress report (1 January - 30 June 1966) are reported herein.

The initiation of an investigation of the effect of sound and/or gas sterilants on the thermophilic bacterium, Bacillus stearothermophilus, are also presented in this report.

2. Effect of propylene oxide and ultrasonic irradiation on spores of B. subtilis var. niger.

In the fourth semiannual progress report (1 January 1966 - 30 June 1966) experiments using propylene oxide levels of 250 and 500 mg/liter were summarized. In the work being presented, further variations in propylene oxide levels and the period of exposure to ultrasonic irradiations were investigated. All data listed represent average figures for at least 4 separate determinations for each parameter listed. Table 1 lists the results obtained when propylene oxide (500 mg/liter) was employed with and without sound against B. subtilis var. niger.

Table 1. Average counts of spores of B. subtilis var. niger exposed to propylene oxide (500 mg/liter) alone, and in combination with ultrasound (34.8 kc/sec) for 100 minutes at 40C and 40% relative humidity.

Without Sound	With Sound
446,000	375

Ultrasonic irradiation distance = 1 inch

These results compare favorably with data previously obtained using a similar level of propylene oxide in combination with ultrasound for exposure periods of 40, 60, 80, and 120 minutes, respectively. The values obtained at 100 minutes as shown in Table 1, are in line with those collected using other periods of exposure.

An exposure period of 100 minutes was also selected in an experiment which employed a propylene oxide level of 1000 mg/liter. The results obtained are shown in Table 2.

Table 2. Average counts of spores of B. subtilis var. niger exposed to propylene oxide (1000 mg/liter) alone, and in combination with ultrasound (34.8 kc/sec) for 100 minutes at 40 C and 40% relative humidity.

Without Sound	With Sound
160	1

Ultrasound irradiation distance = 1 inch.

Comparing the results of Table 1 with Table 2 demonstrates the marked reduction in cell count brought about by the higher level of propylene oxide employed. The effect of ultrasound added to the propylene oxide again illustrates significant kill attributable to the combined agents.

During this period of experimentation, it became necessary to replace the Friedman Horn previously employed with a new one. The new horn was operated at an emission frequency of 33.8 kc/sec. Using the new horn, both statistically significant and insignificant results were obtained. Examples of statistically significant data are shown in

Table 3.in which case propylene oxide was used in a concentration of 1000 mg/liter.

Table 3. Average counts of spores of B. subtilis var. niger exposed to propylene oxide (1000 mg/liter) alone, and in combination with ultrasound (33.8 kc/sec.) for various periods of time at 40C and 40% relative humidity.

Exp. No.	Period of exposure, minutes	Without Sound	With Sound
1	40	71,900	1,629
2	80	237,000	68

Ultrasound irradiation distance = 1 inch.

It is evident from a review of Table 3 that the viable counts obtained, using propylene oxide alone, varied with the experiment. Thus, higher viable counts occurred when propylene oxide was employed singly for 80 minutes rather than at 40 minutes. The distribution of the gas in the test chamber and the possibility of the presence of minute leaks in the system would contribute to the variations in the viable count. In each case, however, the addition of sound to the propylene oxide resulted in significant decreases in cell counts.

Based on previous data, which indicated that ultrasonic irradiations were more effective at lower temperatures, several experiments were planned in which the temperature was decreased to 30C. The results obtained are shown in Table 4.

Table 4. Average counts of spores of B. subtilis var niger exposed to propylene oxide (1000 mg/liter) alone, and in combination with ultrasound (33.8 kc/sec.) for various periods of time at 30C and 40% relative humidity.

Exp. No.	Period of exposure, minutes	Without Sound	With Sound
1	60	27,530	742
2	80	394,000	129

[U]ltrasound irradiation distance = 1 inch.

In both cases, the results obtained were statistically significant which demonstrated that the combined action of propylene oxide and ultrasonic irradiation is desirable.

In order to place the results obtained with propylene oxide in the proper perspective, Table 5 presents a summary of the data collected to date indicating those experiments in which a significance level of 1% was attained using Student's t test. The t values were calculated for populations exposed to propylene oxide in various levels with and without ultrasound.

Table 5. Summary of experiments performed using propylene oxide, in various concentrations, alone and in combination with ultrasound, against B. subtilis var. niger at different temperatures and a relative humidity of 40%.

Period of exposure, minutes	Propylene oxide concentration, mg/liter				
	250	500	1000	1250	
20	◆	-	-	-	
40	■ ◆	□	■	□	
60	■	■	□	■	
80	■	■	▲ ■	□	
100	□	■	▲ ■	□	

- = determination not made

△ = 30 C

□ = 40 C

◇ = 60 C

Solid figures indicate a significance level of 1%

Open figures indicate non-significant t values

Although not all experiments have been completed, as yet, certain generalisations may be tentatively made. Significance levels of 1% were attained more frequently at propylene oxide levels ranging from 250 to 1000 mg/liter than at a propylene oxide level of 1250 mg/liter. At the lowest concentration of propylene oxide (250 mg/liter), and at the highest (1250 mg/liter),

extending the period of exposure to 100 minutes resulted in t values which were not significant. In addition, at a propylene oxide level of 1250 mg/liter, only 25% of experiments performed yielded statistically significant kill due to the combined action of gas plus ultrasonic irradiation. It appears that an optimum level of propylene oxide, to be employed in combination with ultrasonic irradiation, lies in the range of 250 to 1000 mg/liter. The period of exposure of choice seems to vary between 60 and 80 minutes. The most effective temperature to be employed cannot be ascertained until additional experiments employing different temperatures are completed.

3. Sonochemical studies using Bacillus stearothermophilus.

The thermophilic organism, Bacillus stearothermophilus, was selected as a test species because of its unusual temperature tolerance. Initial efforts have been concerned with growing the organism in sufficient quantity and to prepare paper strips impregnated with its spores. Plates were originally determined by the use of Trypticase Soy Agar plates but subsequent work has revealed that Dextrose Tryptone Agar yields more consistent results. To date, spore strips containing an average count of 1.06×10^6 have been prepared. Efforts are now underway to standardize all spore strips and to then carry out experiments designed to investigate the effect of ultrasound on B. stearothermophilus.

4. Future effort.

The combined effects of ultrasonic irradiation and propylene oxide on B. subtilis var. niger at various temperatures will continue. It is

expected that the data collected will allow the selection of optimal conditions for the most effective inhibition of B. subtilis var. niger spores by ultrasound in combination with propylene oxide.

Experiments to determine the effect of ultrasound and/or propylene oxide against B. stearothermophilus will be initiated following a brief study of the effect of temperature on the viability of spores of this microorganism. The data obtained will allow a comparative evaluation of sonochemical sterilization procedures on an additional test organism.

5. Publication.

Work reported previously has been published, the literature citation being as follows:

Pisano, M.A., R.M.G. Boucher, and I.E. Alcamo. 1966. Sterilizing effects of high-intensity airborne sonic and ultrasonic waves. Appl. Microbiol. 14: 732-736.

Five reprints of the above paper have already been distributed to NASA.